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ALLOSTERIC MODULATION OF HAEMOCYANIN OXYGEN-AFFINITY BY L-LACTATE AND URATE IN THE LOBSTER *HOMARUS VULGARIS*

II. CHARACTERIZATION OF SPECIFIC EFFECTOR BINDING SITES

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Summary

The haemocyanin of *Homarus vulgaris* possesses specific binding sites for L-lactate and urate, two allosteric modulators of haemocyanin oxygen-affinity. The affinities for both ligands have been determined. The dissociation constants, K_D, are $0.87\pm0.26\,mmol\,l^{-1}$ for L-lactate and $0.03\pm0.01\,mmol\,l^{-1}$ for urate at 15 °C and pH 8.0. The affinity of the haemocyanin is about 40 times larger for urate than for L-lactate. The stoichiometry of the binding is two ligands per dodecamer in both cases.

L-Lactate does not compete with urate for its binding site and *vice versa*, indicating that the ligand binding sites are independent of each other.

The specificity of urate binding to haemocyanin was investigated in competition experiments with allantoin, caffeine and hypoxanthine. The purine derivatives caffeine and hypoxanthine reduce the binding of urate to haemocyanin, whereas allantoin has no effect. Thus, the purine ring system seems to be essential for the binding of urate to haemocyanin.

Introduction

The oxygen affinity of many arthropod haemocyanins can be modulated by allosteric effectors. Inorganic ions (H^+, Ca^{2+}, Mg^{2+}, Cl^-) as well as organic compounds (L-lactate, urate, dopamine) can affect haemocyanin oxygen-binding characteristics (Truchot, 1975, 1980; Bonaventura and Bonaventura, 1980; Morris *et al.* 1985, 1986; Morris and McMahon, 1989). If a low molecular weight factor is able to modulate the properties of a protein, the presence of a specific binding site must be postulated. According to the MWC model (Monod *et al.* 1965), allosteric modulators bind to the protein at a position different from the active site, resulting in a change of protein conformation.

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In the case of haemocyanin, analysis of effector binding can be carried out in different ways. An indirect method uses oxygen binding curves to study the effect of the modulator *via* its influence on oxygen affinity. The concentration of the protein–ligand complex can be determined directly, using equilibrium dialysis or ultrafiltration techniques. With these direct methods, the determination of the influence of free effector concentration on the amount of ligand bound can be used to estimate the thermodynamic constants of the complex-forming reaction. The affinity of a macromolecule for the ligand is defined as the dissociation constant (K_D). At this effector concentration 50 % of the ligand binding sites are saturated.

To analyse the specificity of the ligand binding site, competition experiments with two ligands can be performed using equilibrium dialysis or ultrafiltration techniques. If both ligands compete for the same binding site, the concentration of the first protein–ligand complex will decrease with increasing concentration of the second ligand. If they bind to different sites on the protein molecule, varying the concentration of one ligand will not influence the amount of the second ligand bound to the protein.

L-Lactate and urate, two organic anions, increase the oxygen affinity of *Homarus vulgaris* haemocyanin (Bridges *et al.* 1984; Bouchet and Truchot, 1985; Taylor and Whiteley, 1989; Bridges, 1990), and their effects have been shown to be additive (Zeis *et al.* 1992). If these organic anions act as modulators of haemocyanin, specific binding sites must be present on the haemocyanin molecule. The characterization of these binding sites for L-lactate and urate on the *Homarus vulgaris* haemocyanin molecule is the subject of the present study. For each ligand, the affinity constant and stoichiometry of the binding to haemocyanin were determined and the interaction of the ligands was studied.

Materials and methods

Five European lobsters (*Homarus vulgaris* Milne-Edwards) were purchased in Roscoff (Brittany, France) and held in the same conditions as previously described (Zeis *et al.* 1992). The concentrations of protein, haemocyanin, L-lactate and urate were measured and the aggregation state of haemocyanin was determined as described by Zeis *et al.* (1992).

Studies on the binding of L-lactate and urate to haemocyanin

Binding experiments were carried out with L-[^{14}C]lactate (150 mCi mmol^{-1}) and [2-^{14}C]urate (55 mCi mmol^{-1}, both Amersham Buchler, Braunschweig, Germany) at 15 °C and pH 8.0. In the first series of experiments, the effect of each ligand was studied separately. Variation of ligand concentration was achieved by adding different concentrations of unlabelled compound to a constant concentration of labelled L-lactate or urate. Subsequently, the concentration of radioactively labelled ligand bound to haemocyanin was measured. For this purpose, two different methods were employed.

Ultrafiltration method

With this technique, haemocyanin together with the bound ligand is separated from the free ligand by ultrafiltration through a membrane impermeable to haemocyanin (Johnson *et al.* 1984). The separation was performed in a special filtration unit (Ultrafree-MC, Millipore, Eschborn, Germany) consisting of an upper chamber with the fixed membrane (molecular weight cut-off 30 000 Da) and a lower chamber. 25 μl of haemolymph was placed in the upper chamber and the whole filtration unit was centrifuged at 11 000 revs min^{-1} (16 000 *g*) for 15 min. The radioactivity in the ultrafiltrate was determined as described below. The ultrafiltration method was used only for the L-lactate binding studies.

Equilibrium dialysis method

In contrast to the technique described above, this method permits the determination of the concentration of bound ligand without separating the protein from the solution containing the free ligand. A membrane (molecular weight cut-off 5000 Da; Medicell Company, London, Great Britain) was placed between two dialysis cells, each having a volume of 250 μl. One cell was filled with haemocyanin solution, the other one was filled with Ringer's solution containing the radioactive and unlabelled ligand at various concentrations (for the composition of the Ringer's solution see Zeis *et al.* 1992). The ligand permeates the membrane until an equilibrium between both cells is reached. Additionally, in the cell containing haemocyanin, an equilibrium is reached between the ligand freely dissolved in the solution and the ligand bound to haemocyanin. The amount of ligand bound to haemocyanin can thus be calculated from the difference in ligand concentration between the cells (Carr, 1961). This method was used for L-lactate and urate binding studies.

For ultrafiltration and equilibrium dialysis experiments, the concentrations of the radioactively labelled L-lactate or urate were determined in a scintillation counter (Beckman, CA, USA). For this purpose, 100 μl (in the equilibrium dialysis experiments) or 20 μl samples (in the ultrafiltration experiments) were added to 5 ml of liquid scintillation cocktail (Rotiszint 2200, Roth, Karlsruhe, Germany). Assuming that radioactive and unlabelled ligand show identical binding characteristics, the total concentration of a ligand can be calculated from the measured concentration of radioactive ligand, since the proportion of labelled to unlabelled ligand is fixed.

Binding data were analysed according to Scatchard (1949). The dissociation constant (K_D) and the number of ligand binding sites per protein molecule (n) were estimated from a plot of the quotient of bound to free ligand (b/f) *versus* bound ligand (b). To compare experiments carried out with different monomeric haemocyanin concentrations ([Hc]), the concentration of bound ligand was standardised according to Chamness and McGuire (1975), allowing us to calculate the term $b_{st}=b/[Hc]$.

The binding data were also analysed according to the Hill equation. The slope of

the regression line provides the value for n_H, an estimation of the cooperativity of ligand binding (Hill, 1910).

Studies on the interaction between the ligands

The effect of urate concentration on the binding of L-lactate to haemocyanin was examined by ultrafiltration experiments. Increasing amounts of unlabelled urate were added to haemocyanin solutions containing a constant L-[^{14}C]lactate concentration. The separation of bound and free ligand and the determination of the L-lactate concentrations were carried out as described above. Three series of ultrafiltration experiments, providing 18 values in triplicate, were carried out.

The effect of L-lactate concentration on the binding of urate to haemocyanin was analysed employing equilibrium dialysis. In this case, for each L-lactate concentration a series with increasing urate concentrations including constant amounts of [^{14}C]urate was carried out. With this experimental design, it was possible to determine the K_D for urate at various L-lactate concentrations.

Studies on the specificity of the urate binding site

To analyse the specificity of the urate binding site, competition experiments were carried out with allantoin, caffeine and hypoxanthine. Caffeine and hypoxanthine are purine derivatives, hypoxanthine being a purine metabolite in crustaceans, whereas caffeine is not present in animals. Allantoin is formed by oxidation of urate in the uricase reaction, in which the purine ring system is opened. A series of four equilibrium dialysis experiments was performed for each analogue. As described above, a constant amount of 2 μmol l^{-1} [2-^{14}C]urate was added to increasing concentrations of one analogue. The concentration of urate bound to haemocyanin was determined as described above.

Results

Studies on L-lactate binding

In equilibrium dialysis, no significant difference in the concentration of the radioactively labelled L-lactate could be measured between the two dialysis cells. With the equilibrium dialysis method, the concentration of bound ligand can be determined without separating the protein from the solution. However, in the case of ligands with a low affinity for haemocyanin, high protein concentrations are necessary to provide measurable differences between the dialysis cells. According to Weber (1975), the protein concentration must be in the range of the dissociation constant of the protein–ligand complex. As the affinity of haemocyanin for L-lactate is low (see below), a high haemocyanin concentration would be necessary to provide measurable differences in equilibrium dialysis. Additionally, in solutions containing high protein concentrations the volume of the protein must be taken into consideration (Achilles *et al.* 1981). The native haemolymph contains about 38 mg ml^{-1} haemocyanin. The ultrafiltration method was the method of choice for studying the binding of this ligand, since this avoids the enrichment of

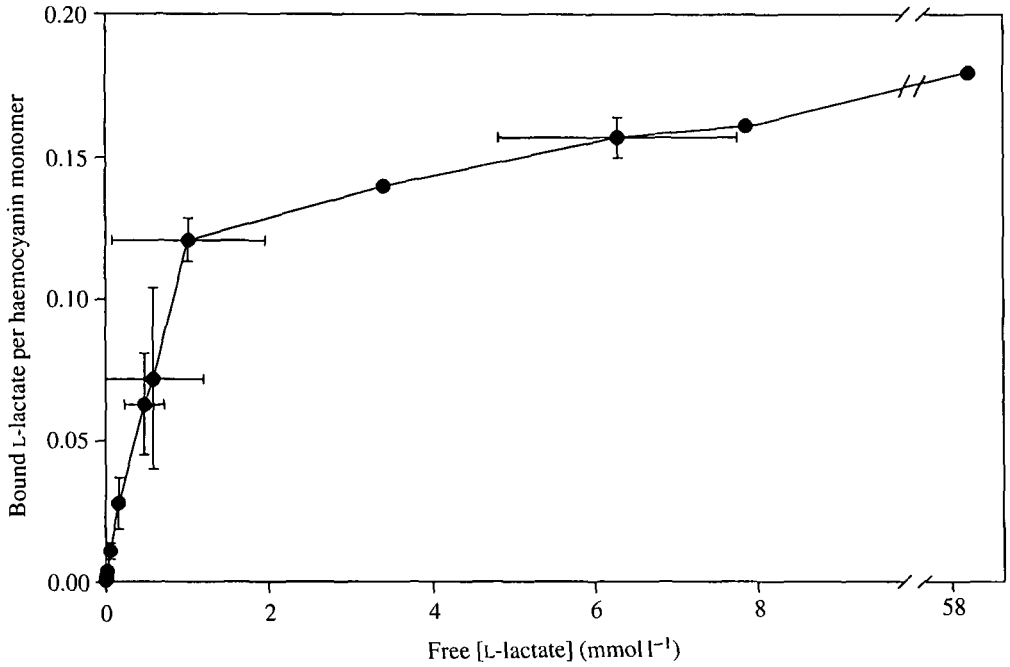

Fig. 1. Binding of L-lactate to haemocyanin. Binding data were obtained by using the ultrafiltration method. The concentrations of bound L-lactate were standardised to the haemocyanin concentration (b_{st}) and plotted *versus* the free L-lactate concentration. The binding curve is hyperbolic, indicating non-cooperative binding. Owing to nonspecific binding, the curve does not reach a plateau, but L-lactate binding is slightly increased even at high L-lactate concentrations. Points without error bars were measured in duplicate only. 15°C, pH 8.0. Error bars are ±S.D., N=8.

haemocyanin to high concentrations, which would be necessary if equilibrium dialysis were used for L-lactate binding studies.

A binding curve for L-lactate to haemocyanin is shown in Fig. 1. The amount of bound L-lactate per haemocyanin molecule increases with rising L-lactate concentrations. From the shape of the curve it is evident that there is a binding site with a high affinity for L-lactate, causing the rapid increase in the amount of bound ligand at low L-lactate concentrations. Because of a low-affinity nonspecific binding site, the amount of bound L-lactate increases with rising concentrations of free L-lactate even beyond the concentration at which the high-affinity binding site is saturated with the ligand.

The binding data were also analysed graphically according to Scatchard (1949), assuming that concentrations exceeding 0.2 mmol l^{-1} L-lactate per mmol l^{-1} haemocyanin monomer represent nonspecific binding of L-lactate to haemocyanin (Chamness and McGuire, 1975). Subtraction of nonspecifically bound ligand from total bound ligand yields the specific L-lactate binding. Using the data shown in Fig. 2, the dissociation constant for L-lactate (K_D) is 0.87±0.26 mmol l^{-1} (N=8) and the number of binding sites (n) is 2.1±0.2 per dodecamer (N=8) at pH 8.0.

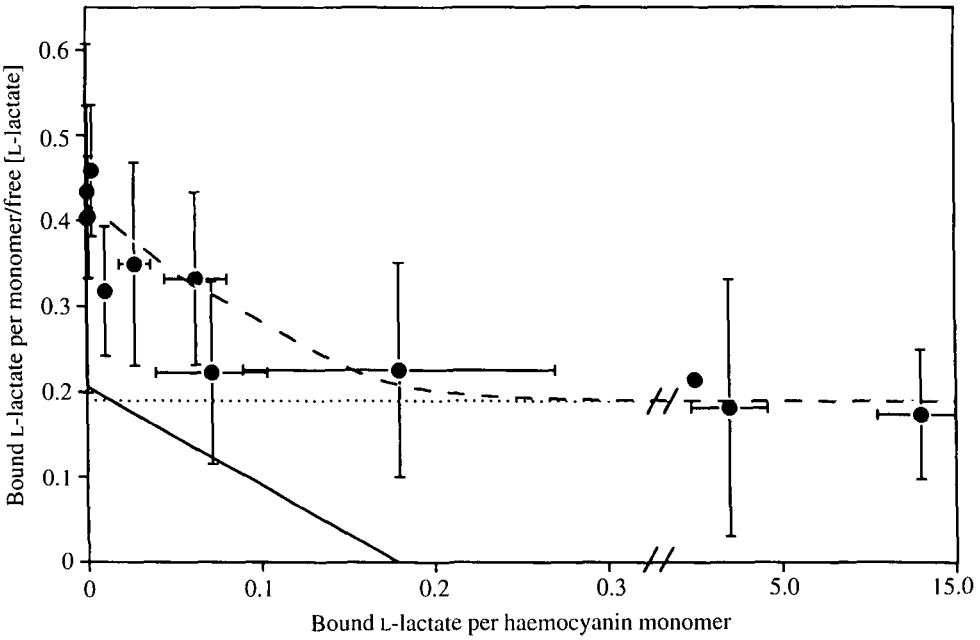

Fig. 2. Binding of L-lactate to haemocyanin. Analysis of the data in Fig. 1 using a Scatchard plot. The value for the dissociation constant K_D and the number of binding sites can be estimated. For this purpose, the binding data (dashed line) are corrected for nonspecific binding (dotted line), yielding specific L-lactate binding (solid line). 15°C, pH 8.0. Values are mean±S.D., N=8.

Estimation of the cooperativity of L-lactate binding to haemocyanin using the Hill equation resulted in a regression line with a slope, n_H, of 0.97, indicating non-cooperative L-lactate binding (Fig. 3).

Studies on urate binding

Because of the higher affinity of haemocyanin for urate it was possible to employ the equilibrium dialysis technique using haemocyanin concentrations in the range of the native concentration. As shown in Fig. 4, the amount of bound urate per haemocyanin molecule (b_{st}) rises hyperbolically with increasing concentrations of free urate. Up to 0.1 mmol l^{-1} free urate, the concentration of bound urate is proportional to the free urate concentration. Between 0.1 and 0.2 mmol l^{-1} free urate, approximately 0.2 mmol urate was bound per mmol monomeric haemocyanin. Above this concentration no further binding occurred, indicating saturation of the binding site.

Urate binding data were also analysed according to Scatchard (1949) and corrected for nonspecific binding (Chamness and McGuire, 1975), which occurs above 0.2 mmol urate per mmol monomeric haemocyanin (Fig. 5). From the regression line, one can estimate the dissociation constant for urate (K_D=0.03±0.01 mmol l^{-1}; N=5) and the number of binding sites on the haemo-

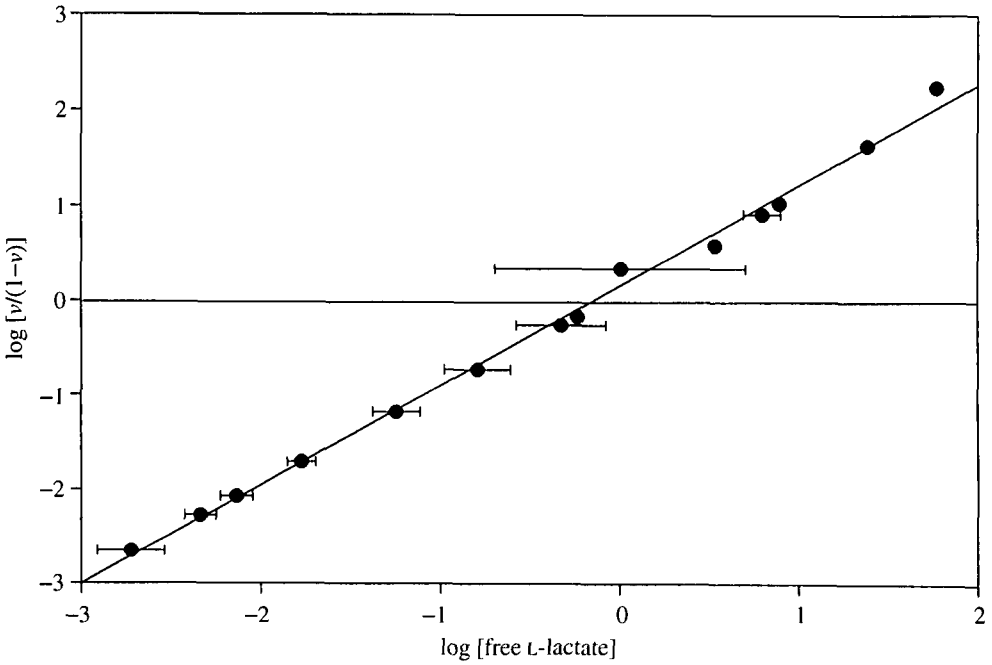

Fig. 3. Binding of L-lactate to haemocyanin. The binding data analysed according to Hill (1910). v is the fractional saturation of haemocyanin with L-lactate. The slope of the regression line provides a value of n_H (0.97), indicating non-cooperative L-lactate binding. 15°C, pH 8.0. Values are mean±S.D., N=8.

cyanin molecule (n=2.0±0.7 per dodecamer; N=5) at pH 8.0. Analysis of urate binding using the Hill equation resulted in a regression line with a slope, n_H, of 0.99 (Fig. 6). The hyperbolic shape of the urate binding curve (Fig. 4) and the value of n_H near 1 indicate non-cooperative binding of urate to haemocyanin.

Interaction of L-lactate and urate binding

Within the urate concentration range between 0 and 0.5 mmol l^{-1}, the presence of urate has no effect on L-lactate binding to haemocyanin (Table 1). The concentration of L-lactate bound to haemocyanin is independent of the urate concentration used.

Corresponding experiments were carried out to analyse the effect of L-lactate concentration on the urate binding to haemocyanin (Table 2). The concentration of urate bound to haemocyanin was not affected by the L-lactate concentration in the range between 0 and 50 mmol l^{-1}. The K_D values for urate binding determined for different L-lactate concentrations are given in Table 2. The affinity of haemocyanin for urate does not alter with increasing L-lactate concentrations.

Specificity of the urate binding site

The specificity of the urate binding site was analysed using equilibrium dialysis for competition experiments with analogues. The competition experiments with

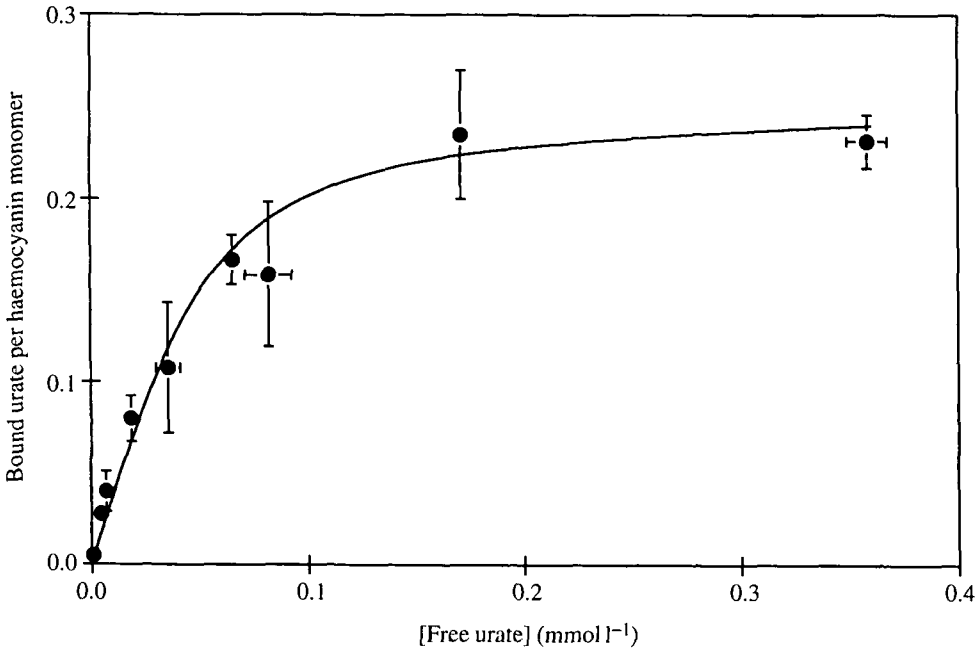

Fig. 4. Binding of urate to haemocyanin. Studies on urate binding were carried out with the equilibrium dialysis technique. The values of bound urate concentrations are standardised to the haemocyanin concentration (b_{st}) and plotted *versus* the concentration of free urate. The curve approaches a limiting value, indicating saturation of urate binding sites on the haemocyanin with ligand. The hyperbolic shape of the curve indicates non-cooperative binding of urate to haemocyanin. 15°C, pH 8.0. Values are mean±S.D., $N=5$.

Table 1. *Binding of* L-*lactate to haemocyanin in the presence of different urate concentrations*

[Urate] (mmol l^{-1})	mmol L-Lactate bound per mmol monomer
0	$7.2\times10^{-4}\pm0.5\times10^{-4}$
0.06	$6.6\times10^{-4}\pm0.6\times10^{-4}$
0.20	$6.4\times10^{-4}\pm0.2\times10^{-4}$
0.27	$6.8\times10^{-4}\pm0.4\times10^{-4}$
0.40	$7.3\times10^{-4}\pm0.7\times10^{-4}$
0.47	$6.9\times10^{-4}\pm0.9\times10^{-4}$

The amount of L-lactate bound per haemocyanin monomer was determined with the ultrafiltration technique ($N=3$ for each urate concentration).

The solution contained 2.3×10^{-3} mmol l^{-1} L-lactate.

The concentration of L-lactate bound to haemocyanin is not changed significantly at any urate concentration studied ($P\leq0.05$).

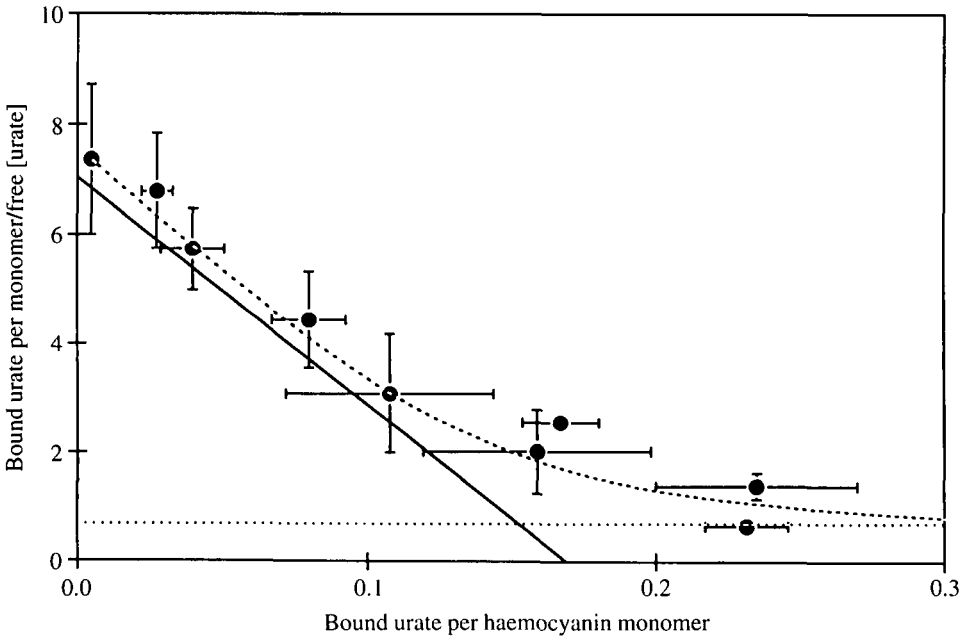

Fig. 5. Binding of urate to haemocyanin. In this Scatchard plot the quotient of the standardised concentration of bound urate (b_{st}) and free urate depend on the concentration of bound urate. The variables K_D and n for urate binding to haemocyanin were derived from the steep part of the curve, which has been corrected for nonspecific binding (dotted line). The corrected regression line is given by the solid line. 15°C, pH 8.0. Values are mean±S.D., N=5.

Table 2. *Binding of urate to haemocyanin in the presence of different* L-*lactate concentrations*

[L-Lactate] (mmol l^{-1})	K_D for urate (mmol l^{-1})
0	0.036±0.010
0.005±0.001	0.039±0.007
0.008±0.001	0.042±0.003
0.047±0.004	0.047±0.006
0.105±0.001	0.046±0.004
0.400±0.200	0.040±0.009
0.900±0.100	0.050±0.011
4.900±0.100	0.041±0.006
50±1	0.043±0.004

Affinity constants of haemocyanin for urate in the presence of different L-lactate concentrations were determined with the equilibrium dialysis technique.

The affinity of haemocyanin for urate is not changed significantly at any L-lactate concentration studied ($P \leq 0.05$).

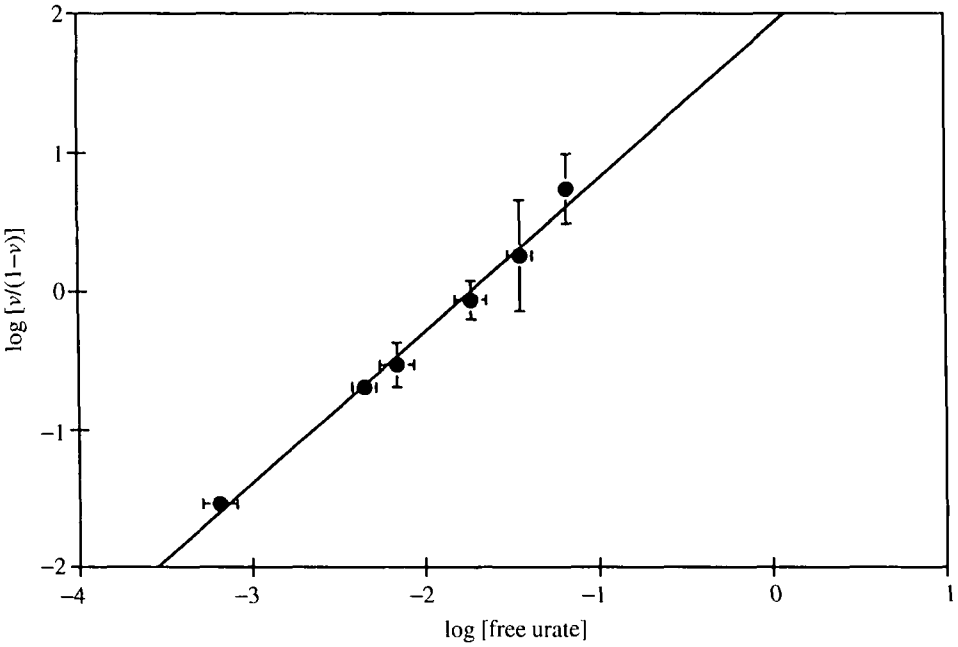

Fig. 6. Binding of urate to haemocyanin. Analysis of the urate binding data with the Hill equation yields a regression line with a slope (n_H) of 0.99, indicating that urate binding to haemocyanin is not cooperative. The urate concentration is in mmol l^{-1}; ν is the fractional saturation of haemocyanin with urate. 15°C, pH 8.0. Values are mean±S.D., N=5.

allantoin show that urate binding to haemocyanin is not influenced by the concentration of allantoin in the range 0–0.5 mmol l^{-1} (Fig. 7). For hypoxanthine, the concentration of urate bound to haemocyanin decreased with rising analogue concentration in the range 0–0.5 mmol l^{-1} hypoxanthine. In the case of caffeine, the effect of the analogue concentration on the urate concentration bound to haemocyanin is more pronounced than for hypoxanthine. A caffeine concentration of 0.25 mmol l^{-1}, more than a hundred times the urate concentration, is able to inhibit urate binding completely.

Discussion

The existence of specific binding sites for L-lactate and urate on the haemocyanin molecule has been demonstrated in the present study.

L-Lactate binding studies

It has been assumed that there is a specific binding site for L-lactate on the haemocyanin molecule ever since Truchot (1980) described its potentiating effect on the oxygen affinity of haemocyanin from *Carcinus maenas* and *Cancer pagurus*. This effect has also been confirmed for *Homarus vulgaris* (Zeis *et al.* 1992).

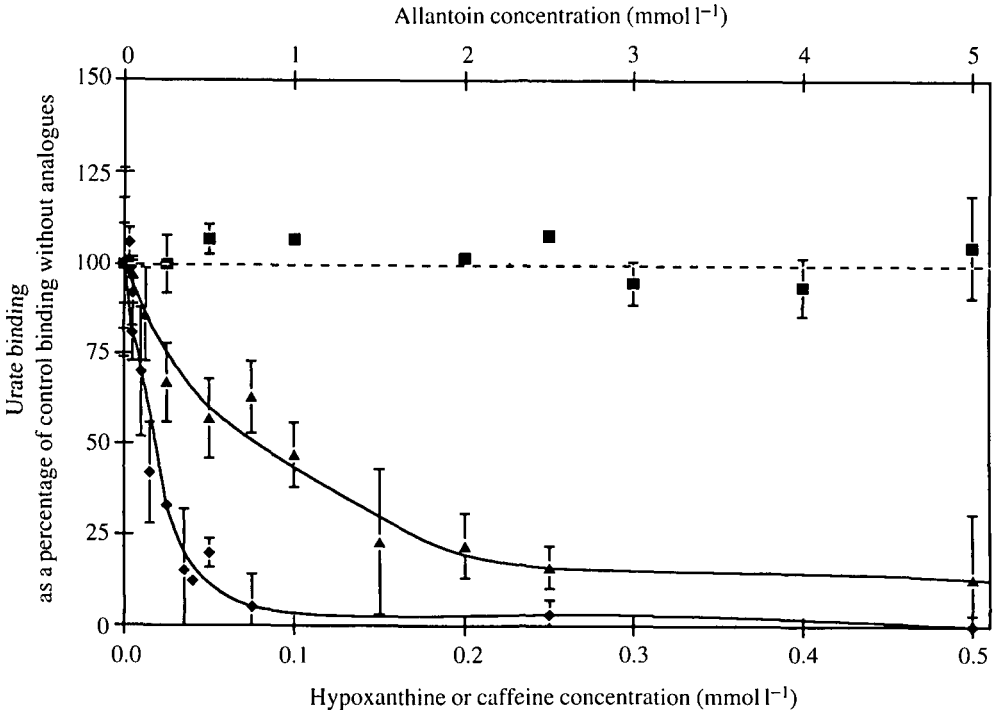

Fig. 7. Specificity of the urate binding site. The effects of allantoin (■), caffeine (◆) and hypoxanthine (▲) on the binding of [^{14}C]urate to haemocyanin were studied using the equilibrium dialysis technique. For each analogue concentration studied, the amount of urate bound to haemocyanin is given as a percentage of the urate binding measured in the absence of the analogue. The concentration of [^{14}C]urate was 0.002 mmol l^{-1} in all experiments. In the presence of caffeine and hypoxanthine, urate binding to haemocyanin is reduced, whereas allantoin has no effect. 15°C, pH 8.0. Values are mean±S.D., N=4.

In the present study, the dissociation constant of the haemocyanin/L-lactate complex in *Homarus vulgaris* was determined by the ultrafiltration technique to be 0.87±0.26 mmol l^{-1}, with two binding sites per haemocyanin dodecamer. Using this technique, Johnson *et al.* (1984) determined a K_D value of 3.2 mmol l^{-1} with 2.8 binding sites per hexamer in *Callinectes sapidus*. A value for K_D of 0.7 mmol l^{-1} and 1.2 binding sites per hexamer in *Panulirus interruptus* was calculated from oxygen binding curves (Johnson *et al.* 1987).

A model of the specific binding site for L-lactate on the haemocyanin molecule was postulated on the basis of oxygen binding curves constructed with L-lactate and its analogues (Graham *et al.* 1983; Johnson *et al.* 1984; Graham, 1985). L-Lactate does not bind to haemocyanin in a cooperative way, although two binding sites exist per dodecamer. Thus, the conformational change in the protein molecule brought about by the first L-lactate molecule bound does not affect the affinity for the second L-lactate molecule.

Urate binding studies

The effect of urate on haemocyanin oxygen-affinity (Morris *et al.* 1985, 1986; Lallier *et al.* 1987; Lallier and Truchot, 1989; Bridges, 1990; Zeis *et al.* 1992) suggests a specific urate binding site on the haemocyanin molecule. The affinity of haemocyanin for urate has been determined for *Homarus vulgaris* in this study using equilibrium dialysis: $K_D=0.03\pm0.01\,mmol\,l^{-1}$ with two binding sites per dodecamer at 15°C and pH 8.0. For the haemocyanin of *C. maenas*, the dissociation constant, K_D, was $0.038\,mmol\,l^{-1}$, and for *P. japonicus* K_D was $0.32\,mmol\,l^{-1}$, with two binding sites per dodecamer in both cases, using an ultrafiltration technique (Lallier, 1988). This author describes the binding of urate to haemocyanin as non-cooperative, which is confirmed for *Homarus vulgaris* in this study. The first urate molecule bound does not alter the affinity of haemocyanin for the second urate molecule.

The analysis of urate binding is limited by the solubility of this purine derivative. Therefore, further experiments with concentrations exceeding $0.5\,mmol\,l^{-1}$ in each dialysis cell cannot be performed with this method. Thus, the nonspecific binding with low affinity is difficult to evaluate. It occurs at high concentrations of urate when the specific binding site is nearly saturated.

Mutual effects of the ligands

The results indicate that L-lactate and urate must have different binding sites on the haemocyanin molecule. Though the stoichiometry is identical in both cases, the affinity of haemocyanin for urate is about 40 times higher than for L-lactate. Binding of either ligand does not affect the affinity of haemocyanin for the other one. The binding sites for L-lactate and urate are independent.

According to the MWC model of allosteric interactions (Monod *et al.* 1965) for haemoglobin, the effect of a positive modulator of oxygen affinity is based on conformational changes in the protein molecule, including changes in the structure of the oxygen binding site. We have shown that L-lactate and urate increase the oxygen affinity of lobster haemocyanin (Zeis *et al.* 1992), suggesting specific binding of both modulators, which has been confirmed in the present study. Furthermore, we have shown their effects to be additive (Zeis *et al.* 1992); thus, the presence of one modulator does not weaken the effect of the second modulator on oxygen affinity, indicating that the conformational changes caused by one modulator do not include the entire molecule, but only a limited environment. The results of this study confirm this assumption, since the binding of L-lactate to haemocyanin does not affect the affinity of haemocyanin for urate. Thus, the conformational change brought about by L-lactate does not alter the structure of the urate binding site. The same is true for the L-lactate binding site, which is not affected by the binding of urate to its specific binding site. Finally, the investigations on cooperativity also support this hypothesis. The cooperativity of oxygen binding does not reach the maximal value possible (Zeis *et al.* 1992). Moreover, the binding of L-lactate and urate show no cooperativity, suggesting

that the binding of the first L-lactate molecule only affects the structure of the part of the protein molecule adjacent to the first L-lactate binding site. The conformational change does not affect the second L-lactate binding site. The same is true for urate.

Specificity of the urate binding site

The competition experiments with urate analogues show that the purine ring system is essential for the binding to the haemocyanin molecule. Allantoin, which does not contain the complete purine ring system, is not able to compete with urate for its binding site (Fig. 7). In contrast, the presence of hypoxanthine and caffeine reduces the amount of urate bound to haemocyanin (Fig. 7). Thus, we propose that the specific urate binding site has no affinity for allantoin, whereas the purine derivatives hypoxanthine and caffeine are able to interact with the urate binding site. The specific binding constants for these analogues should be determined in further studies. Morris *et al.* (1986) have shown that hypoxanthine and caffeine, together with other purine derivatives, increase the oxygen affinity of the haemocyanin of *Austropotamobius pallipes*. This potentiating effect of caffeine and hypoxanthine on the haemocyanin oxygen-affinity can be interpreted as allosteric modulation brought about by their interaction with the urate binding site.

The specificity of the urate binding site does not exclude the binding of any other molecule but urate, but binding seems to be restricted to other purines. From this point of view it is plausible that L-lactate cannot interact with the urate binding site.

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